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TECHNICAL MANUSCRIPT 571

# COMPLEXES OF POLYLYSINE WITH INFECTIOUS VIRAL RNA

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DEPARTMENT OF THE ARMY
Fort Detrick
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# COMPLEXES OF POLYLYSINE WITH INFECTIOUS VIRAL RNA

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Project 1BO61102B71A

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## ABSTRACT

An earlier report showed that polylysine, a synthetic polyamino acid of high molecular weight, reversibly "masked" the infectivity of the infectious ribonucleic acids (IRNA) of Venezuelan equine encephalitis (VEE) and eastern equine encephalitis (EEE) virus and protected the IRNA against inactivation by pancreatic ribonuclease. Results of further studies reported here indicate more directly that the polylysine and RNA form some kind of strong nucleoprotein-type complex. In sucrose gradient centrifugation studies, IRNA alone and complexed polylysine-IRNA had different sedimentation patterns. Data from chromatographic studies with complexed nucleic acids indicated that the polylysine and RNA were firmly bound. Results from investigations that employed other polylysine preparations of different molecular weights, 3,000 to 100,000, showed that polylysine of low molecular weight (3,000) did not block infectivity or protect IRNA against nuclease inactivation. These results suggest that polylysine-IRNA complexes in which infectivity is masked may have a different physical configuration from that of IRNA alone.

## I. INTRODUCTION*

Because of the obvious importance of nucleoproteins in genetic and immunological processes, studies in our laboratories have included examination of complexes formed by viral nucleic acids with basic proteins or polyamino acids. An earlier report[1] showed that polylysine of high molecular weight reversibly "masked" the infectivity of infectious ribonucleic acids (IRNA) from Venezuelan equine encephalitis (VEE) and eastern equine encephalitis (EEE) virus. Results of further studies reported here indicate more directly that polylysine and IRNA form some kind of nucleoprotein-type complex. The physical nature of such complexes was compared with that of IRNA alone in sucrose gradient centrifugation and chromatographic studies.

## II. MATERIALS AND METHODS

Virus source material used in these studies was VEE virus, Trinidad strain, described by Hardy[2] and EEE virus, CDC strain SC7. VEE virus was propagated in monolayer cultures of infected chick fibroblast (CF) or L cells, and EEE, in CF cultures only. Virus was harvested from the supernatant growth medium of the infected cultures and partially purified by one cycle of low- and high-speed centrifugation.

IRNA was extracted from the virus with hot phenol[3] in the presence of ethylenediaminetetraacetate (EDTA) and sodium dodecyl sulfate. The IRNA preparations had specific infectivities of at least $1 \times 10^5$ plaque-forming units/μg of RNA. RNA was determined quantitatively by measuring the optical density of concentrated samples at 260 mμ. Infectivity was assayed by plaque formation in monolayer cultures of CF cells that were pretreated with hypertonic NaCl.[3] The diluent for IRNA in the assays was 0.15 M NaCl, Tris-HCl buffered to pH 8.2.

To demonstrate the residual integrity of the infectious unit in IRNA that was complexed with high molecular weight polylysine, such preparations were treated with pronase to digest the polylysine. Complete recovery of infectivity was accomplished in 30 minutes at 37 C when 1 unit of pronase/μg polylysine was used. Because pronase (>0.5 unit/ml) can damage cell cultures used in the assays, it was not possible in some experiments to use the optimal ratio of pronase to polylysine. Therefore, in those cases partial recovery of infectivity was effected.

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Fluorescein-labeled polylysine was prepared with fluoroisothiocyanate by methods[4] used for preparing fluorescent antibody. After the labeling procedure was completed, the product was chromatographed on Sephadex G-25 to remove free dye. No free dye was detected when a sample of the chromatographed product was subjected to dialysis. Fluorescence of samples was determined with a Turner fluorometer using filters providing exciting light peaking at 436 mμ and permitting passing of light above 510 mμ.

Polylysine preparations of 100,000 molecular weight were obtained from Mann Fine Chemicals, Inc.* and the others of lower molecular weight from Miles Laboratories, Inc.**

## III. RESULTS

Differences that were observed[1] between the biological activity of IRNA alone and that of polylysine-IRNA suggested, somewhat indirectly, that some kind of "nucleoprotein" complex was formed. In more recent studies we have tried to determine more clearly whether such complexes could be distinguished from IRNA alone by physical differences.

For sucrose gradient centrifugation studies, a preparation of VEE IRNA was divided into two portions before layering onto two separate gradients. To one half, polylysine of 100,000 molecular weight was added in a ratio of 1:6 polylysine:RNA. Plaque assay determined that 80% of the infectivity of this complexed preparation was masked. The second half of the preparation was diluted with phosphate buffer to the same concentration of RNA as contained in the complexed preparation. The samples of IRNA alone and of polylysine-IRNA were placed on separate gradients of 10 to 28% sucrose in 0.02 M phosphate buffer, pH 7.4, containing $10^{-3}$ M EDTA. The gradients were centrifuged for 3.5 hours at 57,000 x g in an SW 39 rotor of a Spinco Model L centrifuge. Fractions were collected dropwise, six drops per fraction, from the bottom of the tubes. These fractions were assayed for infectivity; fractions from the polylysine-IRNA were assayed both before and after pronase treatment to digest the polylysine. Data from this study, summarized in Figure 1, indicate that IRNA in the presence of polylysine sedimented more rapidly than control IRNA alone. Infectivity of the fractions is expressed as percentage of the total infectivity recovered from each centrifuged sample. Polylysine alone (not shown in Figure 1) centrifuged under the same conditions remained in the top two fractions of the gradient. In the case of polylysine-IRNA, 100 times as much infectivity was recovered from the bottom quarter of the gradient (greater than 23% sucrose) as was the case with control IRNA. Total infectivity of fractions from the centrifuged polylysine-IRNA after pronase treatment was approximately twice that of the untreated fractions,

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and most of this increase was observed in the lower fractions of the gradient. The data shown also suggest that some complexes of IRNA and polylysine remain infectious, i.e., even without pronase digestion significantly more infectivity was observed in lower fractions of centrifuged polylysine-IRNA than in similar fractions of centrifuged control IRNA alone.

Detection and quantitation of polylysine was of interest in studies such as those just described, but none of the chemical analytical methods tested gave sensitive enough results. Recently, we have been able to detect and roughly quantitate polylysine by use of fluorescein-labeled polylysine. The dye-labeled polyamino acid retained the capacity to mask infectivity of IRNA reversibly. In initial tests to examine the capabilities of the labeled product, prepared with polylysine of 100,000 molecular weight, it was used in chromatographic experiments with RNA from normal chick embryo. Three chromatographic runs were made using a 0.9 cm by 36 cm column of Sepharose 4B. Phosphate buffer 0.02 M, pH 7.4, containing $10^{-3}$ M EDTA, was used to prepare the column and to elute the samples. Free dye was removed from the labeled polylysine by chromatography on Sephadex G-25. The three samples chromatographed were: (i) fluorescein-labeled polylysine alone, (ii) RNA alone, and (iii) polylysine-RNA formed with a ratio of 1:6 dye-labeled polylysine:RNA. The amount of RNA or polylysine used in each experiment was the same. Data from the two control experiments, with RNA or polylysine alone, are presented together in Figure 2. As seen in the figure, the RNA was eluted in a single peak immediately after the void volume. The apparent trailing may be due to different species of cellular RNA that differ in molecular size. Fluorescence observed in fractions eluted when the labeled polylysine alone was chromatographed indicated that the polylysine was retarded on the column, although the total fluorescence observed in the eluted fractions was much less than expected. It was discovered later that a visible amount of dye remained at the top of the column. This may be explained by an observation by Andrews[5] that agar gels may act as cation exchangers and retain basic substances. Nevertheless, it appears that free polylysine and free RNA would be separated on Sepharose 4B even though some of the polylysine might be retained on the column.

When polylysine and RNA were mixed before they were placed on the column and the eluted fractions assayed both for fluorescence and for UV absorption at 260 mμ, the elution patterns for RNA and polylysine were those shown in Figure 3. Much more fluorescence was observed in these eluted fractions than in the experiment with control labeled polylysine, indicating that polylysine was not retained on the column when the complex was chromatographed. The similarity of the elution patterns for polylysine and RNA in this experiment is strong evidence for complex formation. No difference was detected between the elution patterns for RNA alone or for the complex in these experiments. However, further studies are planned for chromatographing complexes of IRNA both on Sepharose 2B that acts as a molecular sieve for larger molecules and on $CF_{11}$ cellulose by the method of Franklin[6] that separates RNA of different secondary structures.

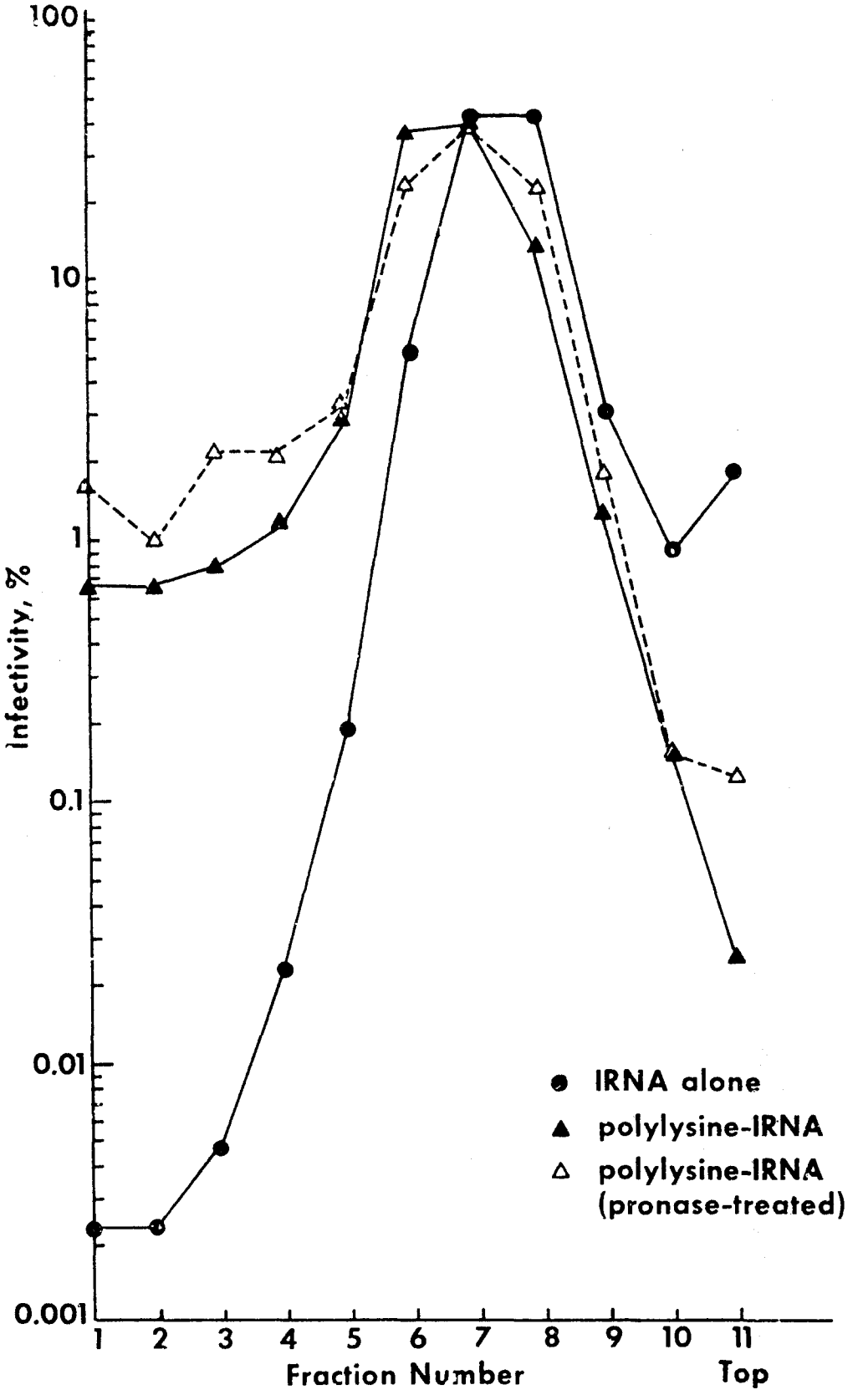


FIGURE 1. Sucrose Cradient Centrifugation of Polylysine-IRNA.

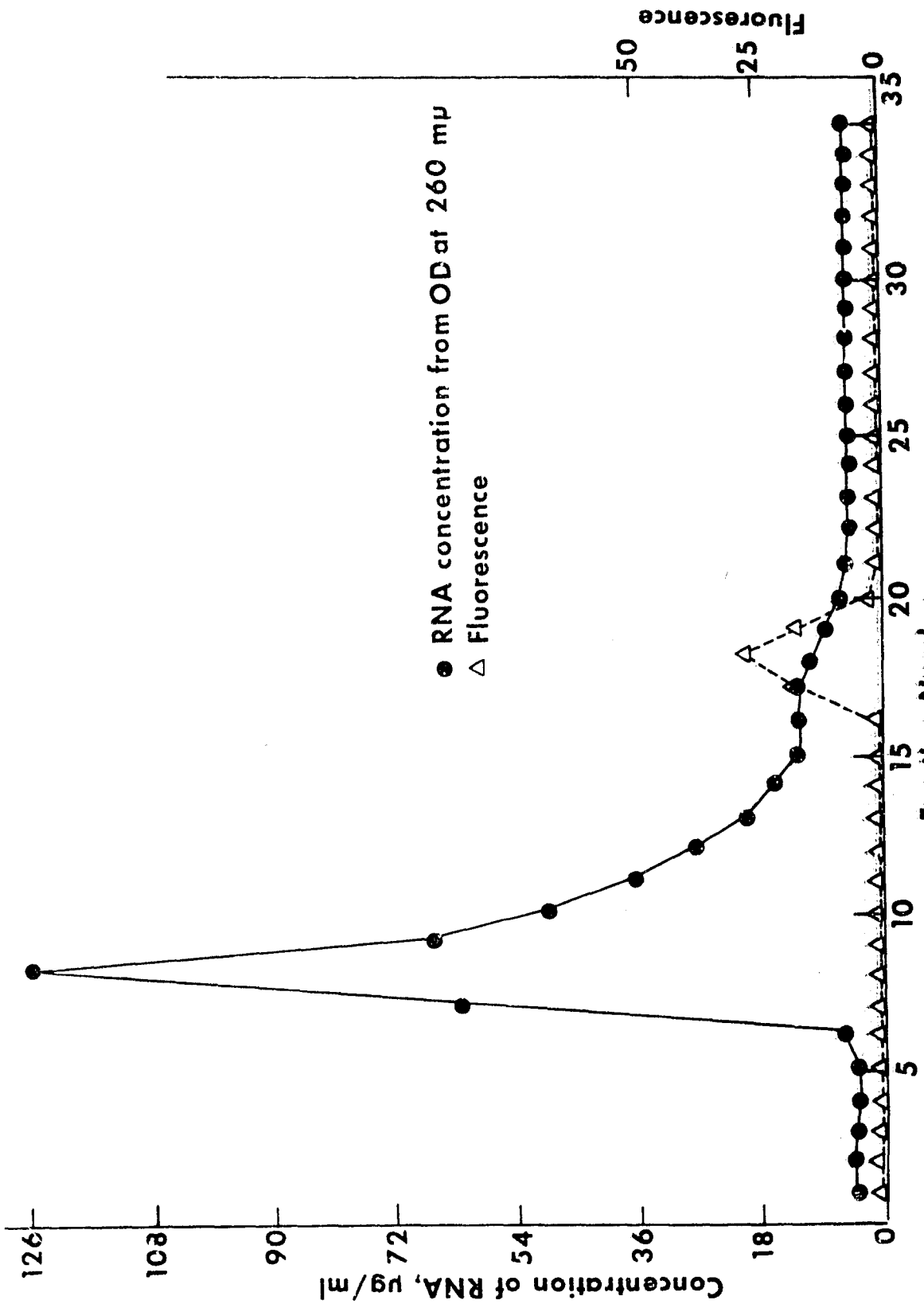


FIGURE 2. Chromatography of Control RNA and of Polylysine Alone.

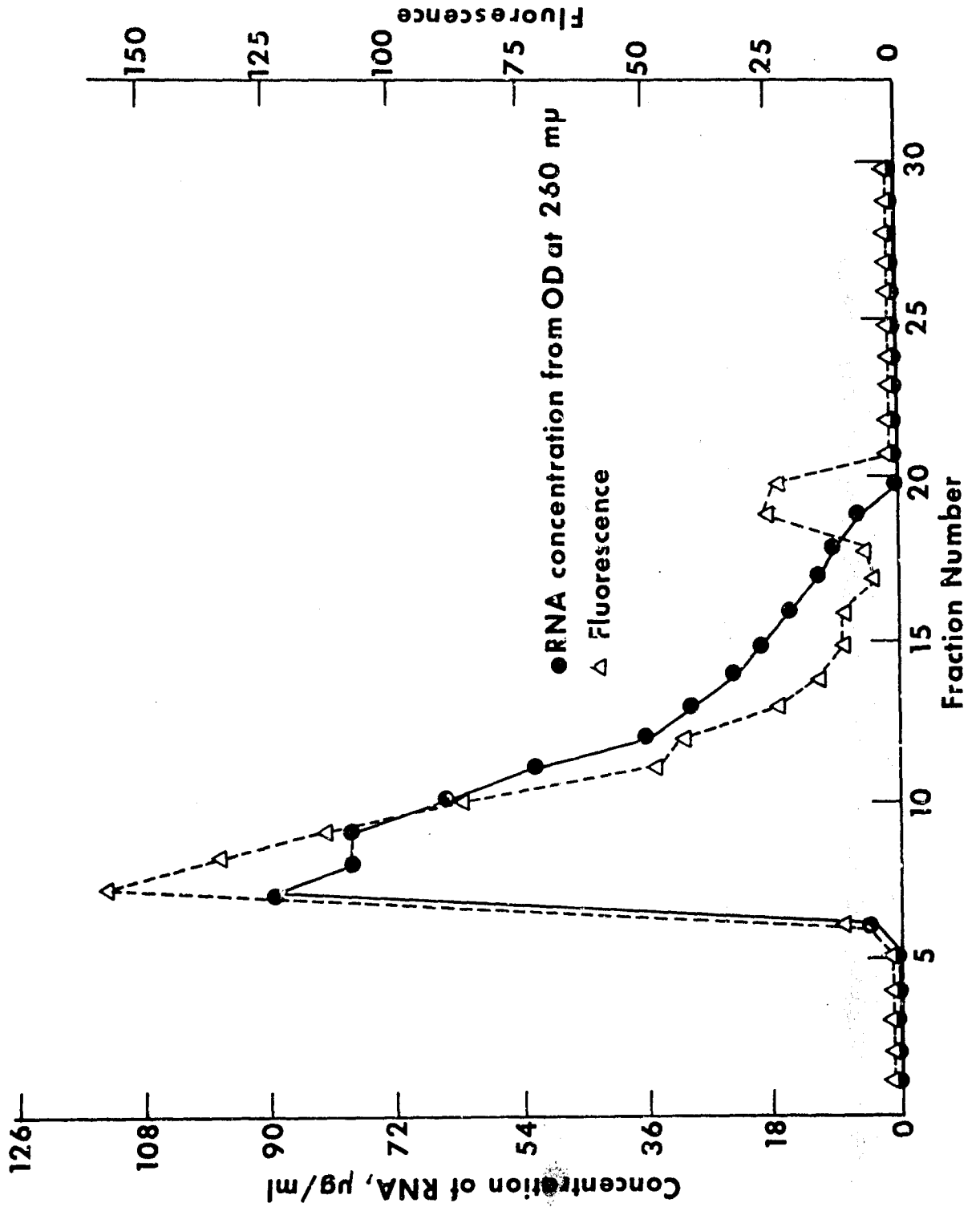


FIGURE 3. Chromatography of Complexed Polylysine-RNA.

In other studies with EEE IRNA we examined whether blocking of infectivity was dependent on the molecular size of the polylysine. Commercial preparations of four different molecular weight values were used in the investigations. These values ranged from 3,000 to 100,000. All preparations were used at the same concentration by weight. The concentration selected was one predetermined to result in >99% masking of the infectivity when polylysine of 100,000 molecular weight was used. Each of the preparations in appropriate concentration was added to a separate sample of EEE IRNA. The mixtures were assayed before and after pronase treatment. Data from a representative experiment (Table 1) indicated that polylysine preparations of molecular weight 44,000 to 100,000 were equally effective in reversibly masking infectivity. However, the preparation of lower molecular weight, 3,000, did not significantly affect infectivity when present at the same concentration as polylysine of effective molecular weight. The lower molecular weight material will be examined at higher concentrations, and when available, material of molecular weights between 3,000 and 44,000 will also be studied.

TABLE 1. EFFECT OF MOLECULAR WEIGHT OF POLYLYSINE ON REVERSIBLE MASKING OF INFECTIVITY OF IRNA

| Sample | Molecular Weight of Polylysine | Titer, $\log_{10}$ pfu/ml | |
|---|---|---|---|
| | | Untreated | Pronase-Treated |
| IRNA alone | - | 5.8 | 5.5 |
| Polylysine-IRNA | 3,000 | 5.5 | 5.1 |
| | 44,000 | <2.7 | 4.8 |
| | 75,000 | <2.7 | 5.0 |
| | 100,000 | <2.7 | 4.9 |

Protection against nuclease inactivation was observed[1] in the presence of polylysine of 100,000 molecular weight. A comparison of the effect of pancreatic ribonuclease on IRNA in the presence of low and high molecular weight polylysine is shown in Table 2. Samples of IRNA alone or in the presence of polylysine were treated for 5 minutes at 37 C with 0.001 or 0.0001 µg/ml of pancreatic ribonuclease. Samples containing the high molecular weight polylysine were subsequently treated with pronase to digest the polyamino acid. Appropriate control samples without ribonuclease were incubated under the same conditions. Immediately after incubation, samples were diluted and assayed for infectivity. As reported previously,[1] no inactivation of IRNA was observed when complexes of polylysine-IRNA, prepared with high molecular weight polylysine, were treated with pancreatic ribonuclease. However, in the presence of low molecular weight polylysine, nuclease inactivation of IRNA was similar to that of uncomplexed IRNA. This was true for less strenuous ribonuclease treatments as well as for the 99% inactivation shown here.

TABLE 2. COMPARISON OF LOW AND HIGH MOLECULAR WEIGHT POLYLYSINE FOR EFFECT ON RIBONUCLEASE SENSITIVITY OF IRNA

| Sample | Molecular Weight of Polylysine | Inactivation by Ribonuclease, %[a] |
|---|---|---|
| IRNA alone | - | 99 |
| Polylysine-IRNA | 100,000 | 0 |
| Polylysine-IRNA | 3,000 | 99 |

a. 0.001 μg/ml.

## IV. DISCUSSION

Data from studies reported here indicate that IRNA does form strong complexes with polylysine. Information from sucrose gradient studies indicates that IRNA may form complexes that are infectious as well as those in which the infectivity is masked. It is possible that masking of infectivity may occur only when some required proportion of polylysine is contained in the complex, or that attached polylysine is capable of masking infectivity only when attached in some particular fashion to the IRNA. Observations with low molecular weight polylysine may support the latter supposition; i.e., it seems likely from the basicity of lysine that this low molecular weight material must be capable of binding to the IRNA and yet does not affect infectivity or protect the IRNA against nuclease inactivation. This might be because the lower molecular weight material can attach to the IRNA without altering its physical structure, while the higher weight material may enforce a different molecular configuration on the nucleic acid.

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13. ABSTRACT

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